JOURNAL OF CLINICAL MICROBIOLOGY, May 1995, p. 1371–1374 Vol. 33, No. 5
0095-1137/95/$04.00+0

Differentiation of Pathogenic *Escherichia coli* Strains in Brazilian Children by PCR

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Received 1 December 1994/Returned for modification 27 January 1995/Accepted 16 February 1995

A PCR technique to differentiate pathogenic enteric *Escherichia coli* strains in a field setting was evaluated. Among 76 children with acute diarrhea, this technique identified 12 children (16%) with enterotoxigenic *E. coli*, 6 (8%) with enteropathogenic *E. coli*, and 1 (1%) with enteroinvasive *E. coli* infection. Compared with the conventional assays, the PCR method proved to be simpler, more rapid, and inexpensive and therefore suitable for application in a developing-country field setting.

Diarrheal disease remains a major public health problem in developing countries (25). *Escherichia coli* strains are among the most important bacterial causes of childhood diarrhea. At least five categories of diarrheagenic *E. coli* strains are recognized on the basis of distinct epidemiological and clinical features, specific virulence determinants, and association with certain serotypes (12, 15). Because of the costly and labor-intensive diagnostic procedures, the epidemiology of *E. coli* infections remains obscure in many parts of the world. This study was undertaken to evaluate the application of a PCR-based test to differentiate *E. coli* isolates and determine their distribution among children with and without diarrhea.

The study was conducted at Hospital Infantil of the Federal University of Bahia in Salvador de Bahia, Brazil. From 1 June to 31 August 1993, all children under 5 years of age with acute diarrhea who were brought to the hospital ambulatory clinic in the afternoon, Monday through Friday, were enrolled in the study.

Two rectal swabs were collected from each child, placed in Cary-Blair transport medium, and processed within 4 h. One swab was processed by routine microbiological and biochemical tests to identify *E. coli*, *Salmonella* spp., *Shigella* spp., and *Campylobacter jejuni*, while the second swab was stored in 2 ml of phosphate-buffered saline (pH 7.4) at 4°C until tested for rotavirus by enzyme immunoassay (EIA) (LMD Laboratories, Inc., Carlsbad, Calif.). Fecal samples and/or rectal swab specimens were obtained for detection of *Cryptosporidium parvum* by enzyme-linked immunosorbent assay (ELISA) (Alexon Inc., Sunnyvale, Calif., and LMD Laboratories, Inc.).

Three to six lactose-fermenting colonies and up to three lactose-negative colonies from each child were selected from MacConkey plates to be tested by conventional and PCR procedures. A total of 239 isolates were obtained from the 76 children and stored on tryptic soy agar (Difco Laboratories, Detroit, Mich.) gridplates for later testing by reference virulence assays. In addition, 43 isolates from 16 children without diarrhea were tested as controls. All conventional virulence assays were performed in the research laboratory at Cornell University Medical College, New York, N.Y. Gridplates containing the *E. coli* strains were transported from Brazil and stored at 4°C until processed. All specimens were processed within 4 months of the date of collection.

Bacterial colonies for each patient were pooled for template DNA preparation immediately prior to PCR testing, suspended in 300 μl of sterile distilled water, and boiled for 10 min. A 10-μl aliquot of this suspension was added to 90 μl of PCR mixture (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate [Pharmacia Biotech Inc., Piscataway, N.J.], and 2.5 U of *Taq* polymerase [Boehringer Mannheim Biochemicals, Indianapolis, Ind.]) and subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min, and extension at 72°C for 1 min. The amplified DNA products were resolved by agarose gel electrophoresis and visualized by UV transillumination after ethidium bromide staining.

Oligonucleotide primers used to detect enterotoxigenic, enteropathogenic, and enteroinvasive *E. coli* (ETEC, EPEC, and EIEC, respectively) by PCR tests are shown in Table 1. Enterohemorrhagic *E. coli* strains were not looked for in this study as they are not believed to play a significant role in the etiology of pediatric diarrhea in the region studied (8). The specific adherence patterns of enteroaggregative and diffuse adherent *E. coli* were detected by HeLa cell adherence assay (19).

Reference strains used as positive controls in the PCR tests included the EPEC strain B171 (O111:NM), the heat-stable enterotoxin (STa)-producing ETEC strain TX-1 (O78:H12), the heat-labile enterotoxin (LT)-producing ETEC strain E2539-C1 (O25:NM), the LT- and STa-producing ETEC strain EDL1493 (O6:H6) (kindly provided by Joy Wells, Centers for Disease Control and Prevention, Atlanta, Ga.), and the EIEC strain E11 (obtained from Stanley Falkow, Stanford University, Stanford, Calif.). Each strain could be identified by its distinct amplicon size (Fig. 1). The nonpathogenic *E. coli* strains K-12 (NX185) and HB101 were used as negative controls and to monitor for PCR contamination. Standard precautions against carryover contamination were used (16).

The production of LT by *E. coli* was determined by the GM1-ganglioside ELISA procedure as previously described (17, 23). Samples were pooled and tested in duplicate. The ETEC STa was detected with a commercially available EIA-based kit (Oxoid, Hampshire, England) according to the manufacturer's instructions.

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TABLE 1. Oligonucleotide primers

Target gene (enteropathogen)	Primer designation and sequence	Location (bp) from 5′ end	Amplicon size (bp)	Reference(s)
BFP gene (EPEC)	EP1, 5′CAATGGTGCTTGCGCTTGCT3′	119–138	324	4
	EP2, 5′GCCGCTTTATCCAACCTGGT3′	443–422		
LT gene (ETEC)	ET-LT1, 5′GCGACAAATTATACCGTGCT3′	59–76	708	26
	ET-LT2, 5′CCGAATTCTGTTATATATGT3′	765–746		
STa gene (ETEC)	ET-ST1, 5′CTGTATTGTCTTTTTCACCT3′	79–98	182	5, 14
	ET-ST2, 5′GCACCCGGTACAAGCAGGAT3′	260–241		
ipaH (*Shigella* spp. and EIEC)	EI-1, 5′GCTGGAAAAACTCAGTGCCT3′	1061–1080	424	24
	EI-2, 5′CCAGTCCGTAAATTCATTCT3′	1484–1465		

Toxin-producing strains as determined by ELISA and PCR were then retested by DNA hybridization with the specific toxin gene probes. These DNA probes were generated by labeling the PCR products obtained with the LT and STa primers respectively, with a digoxigenin labeling kit (Boehringer Mannheim). Hybridization was performed under high-stringency conditions (18), and the signals were visualized by chemiluminescence (Boehringer Mannheim).

A total of 232 *E. coli* isolates from 76 children with diarrhea and 16 children without diarrhea were tested individually by the HeLa cell adherence assay as described previously (3, 19). The remaining isolates could not be tested in this assay because of storage plate contamination.

The PCR tests detected 12 (16%) ETEC (LT- and/or STa-PCR-positive) infections, 6 (8%) EPEC (bundle-forming pilus [BFP]-PCR-positive) infections, and 1 (1%) EIEC (*ipaH*-PCR-positive) infection among children with acute diarrhea (Table 2). Only one *E. coli* isolate from a control child yielded a positive result by both the LT and the STa PCR tests for ETEC. This isolate was also positive by GM1-ganglioside ELISA.

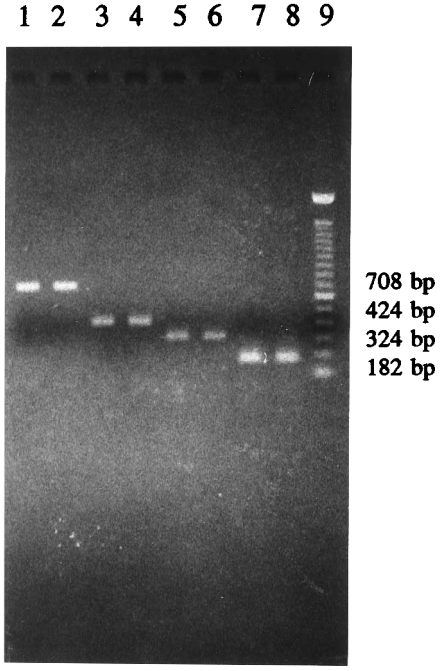

FIG. 1. Agarose gel electrophoresis of PCR-amplified DNA products. Lanes 1 and 2, LT-PCR (708 bp); lanes 3 and 4, *ipaH* PCR (424 bp); lanes 5 and 6, BFP-PCR (324 bp); lanes 7 and 8, STa-PCR (182 bp); lane 9, molecular size marker (100-bp ladder; Gibco BRL).

Both the LT- and the STa-PCR results correlated well with the gene probe DNA hybridization analysis (Table 3). Of the nine strains detected by the LT-PCR test (two by LT-PCR primers and seven by LT- and STa-PCR primers), six were gene probe positive (two of nine strains were no longer viable and hence not tested). The STa gene probe hybridized to 7 of 11 PCR-positive samples (four STa and seven LT and STa genotypes) (3 of 11 strains were not viable). One sample repeatedly amplified by both the LT- and the STa-PCR primers failed to hybridize with either the LT or the STa gene probe.

LT-ELISA-positive strains were isolated from eight children with diarrhea and two children without diarrhea. Of these, two strains were amplified by LT-PCR primers, whereas the remaining six ELISA-positive samples were negative by repeat LT-PCR tests. Two other ELISA-positive isolates hybridized with the LT gene probe. We did not identify any STa-producing *E. coli* with the *E. coli* STa EIA kit (Table 3).

EPEC infections were detected in six children with diarrhea by PCR amplification using primers derived from the BFP gene sequence of EPEC (4, 7). The same six strains were also identified by their characteristic localized adherence to HeLa cells. Diffuse adherent *E. coli* strains were found in 15 cases and 4 controls, and enteroaggregative *E. coli* strains were detected in 6 cases and 3 controls.

Among the 76 children with acute diarrhea, *Salmonella* spp. (12%), *Shigella* spp. (3%), and rotavirus (30%) infections but no *C. jejuni* infections, were identified. The two *Shigella* sp. isolates identified biochemically were detected by the PCR primers designed to detect EIEC. Mixed infections were present in 22 (29%) of these children. Cryptosporidial antigen was detected in 12 (19%) of 64 samples obtained from symptomatic children. Using the PCR method, we detected pathogenic *E. coli* (ETEC, EPEC, and EIEC) strains in the fecal samples of 19 children with acute diarrhea (25%), whereas only one asymptomatic child was found to harbor an ETEC strain with an LT-STa-PCR-positive genotype (Table 2).

Using a DNA probe derived from the BFP structural gene,

TABLE 2. *E. coli* enteropathogens isolated from children with or without diarrhea as detected by PCR tests

Pathogen and PCR type	Total no. of patients (%) positive	
	With diarrhea (n = 76)	Without diarrhea (n = 16)
ETEC	12 (16)	1 (1)
LT	2 (3)	0
STa	4 (5)	0
LT-STa	6 (8)	1 (1)
EPEC, BFP	6 (8)	0
EIEC, *ipaH*	1 (1)	0
Total	19 (25)	1 (1)

TABLE 3. Comparison of PCR tests with ELISA and toxin gene probe DNA hybridization for the detection of ETEC infections[a]

Method (*n*)	No. of correlating results obtained by:				
	PCR			Gene probe	
	LT (*n* = 2)	STa (*n* = 4)	LT-STa (*n* = 7)	LT (*n* = 8)	STa (*n* = 7)
LT-ELISA (10)	0	ND[b]	2	2	ND
STa-EIA (0)	ND	0	0	ND	ND
LT gene probe (8)	1[c]	ND	5[c]		
STa gene probe (7)	ND	2[d]	5[c]		

[a] *n*, total number of strains positive by the method indicated.
[b] ND, not done.
[c] One strain not viable.
[d] Two strains not viable.

Giron et al. had shown that this probe detected EPEC with a higher specificity than did the enteroadherence factor probe (6). In a further application, a PCR assay using oligonucleotide primers derived from the BFP sequence of EPEC proved to be not only specific for the detection of localized-adherence-associated EPEC but also quite simple and rapid (10). In this field study, all strains that exhibited localized adherence in the HeLa cell assay were detected by the BFP-PCR test.

Furthermore, our findings demonstrate the previously unrecognized importance of ETEC as a cause of childhood diarrhea in the part of Brazil studied. The PCR and ELISA results did not correlate well, however. The DNA of two gene probe- and ELISA-positive strains was not amplified by LT-PCR. This may be due to mutations or deletions at the primer binding sites in the target gene (22). At the same time, the LT gene probe failed to detect two LT-PCR- and ELISA-positive samples, indicating a limited sensitivity of the DNA hybridization method (22). The remaining ELISA-positive, PCR-negative, and probe-negative strains may represent false-positive results due to the presence of a product similar to LT in its binding and antigenic characteristics (17).

The commercially available competitive EIA for the detection of STa did not detect STa production by any of our STa-PCR-positive strains. This may reflect an increased sensitivity of the PCR compared with that of the EIA or may indicate that the toxin was not expressed under in vitro conditions (1, 9, 15, 20, 22). This is supported by the observation that 7 of 11 STa-PCR-positive strains were also detected by DNA hybridization (three strains were not viable). The fact that one sample was repeatedly amplified by both LT- and STa-PCR primers but did not hybridize to LT and STa gene probes may again reflect the limited sensitivity of the DNA hybridization method.

We diagnosed only one EIEC infection by PCR using *ipaH*-derived primers and believe that EIEC strains are relatively uncommon in the part of Brazil studied and in other parts of South America (13). This PCR technique had previously been found to be very sensitive (21).

The introduction of PCR technology into the local laboratory has considerably simplified the detection of *E. coli* associated with diarrhea. The sample DNA preparation was simply reduced to boiling bacteria in water for 10 min. In contrast to the reference assays, the DNA for PCR can be prepared from dead as well as contaminated organisms. As indicated above, we were able to detect pathogenic *E. coli* strains from plates that became contaminated or nonviable. Samples can be processed within 1 day after collection and overnight growth, with a fraction of the labor and cost necessary for conventional assays. Cell culture facilities, which are expensive and require research laboratory settings, are not required.

Some investigators have attempted to extract DNA directly from stool samples for PCR tests, but these methods are time-consuming and the results may be unreliable (2, 11). Furthermore, the high level of sensitivity of direct PCR may not correlate well with clinical manifestations (5, 21). The PCR application in this study does not change the sensitivity of detection of the pathogenic *E. coli* strains, since the colonies for the test are first selected from MacConkey plates, just as they are selected for the standard biochemical tests. Hence, the usual correlation of the number of colonies associated with clinical manifestations of diarrhea caused by *E. coli* is preserved.

We believe that this procedure can be used in many developing-country settings to increase our understanding of the epidemiology of *E. coli*-associated diarrhea.

We thank Heonir Rocha and Luciana Silva of the Federal University of Bahia, and M. Altwegg of the University of Zürich, Zürich, Switzerland, for their helpful advice; Brazilian medical students Cláudia D. Macédo, Christiane M. F. Pinto, João J. B. de Barros dos Santos, and Alfonso J. B. Cerdeira for their invaluable introduction into the field; Chris Johnson for his laboratory support; and the companies Alexon and LMD Laboratories, Inc., for donation of the cryptosporidium and rotavirus EIAs.

This work was supported by grants from the Cornell Scholars Program in Biomedical Sciences and the Deutsche Forschungsgemeinschaft, Germany.

REFERENCES

1. **Blomen, I., S. Löfdahl, T. A. Stenström, and R. Norberg.** 1993. Identification of enterotoxigenic *Escherichia coli* isolates: a comparison of PCR, DNA hybridization, ELISAs and bioassays. J. Microbiol. Methods **17:**181–191.
2. **Brian, M. J., M. Frosolono, B. E. Murray, A. Miranda, E. L. Lopez, H. F. Gomez, and T. G. Cleary.** 1992. Polymerase chain reaction for diagnosis of enterohemorrhagic *Escherichia coli* infection and hemolytic-uremic syndrome. J. Clin. Microbiol. **30:**1801–1806.
3. **Cravioto, A., R. J. Gross, S. M. Scotland, and B. Rowe.** 1979. An adhesive factor found in strains of *Escherichia coli* belonging to the traditional infantile enteropathogenic serotypes. Curr. Microbiol. **3:**181–184.
4. **Donnenberg, M. S., J. A. Giron, J. P. Nataro, and J. B. Kaper.** 1992. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. Mol. Microbiol. **6:**3427–3437.
5. **Frankel, G., J. A. Giron, J. Valmassoi, and G. K. Schoolnik.** 1989. Multi-gene amplification: simultaneous detection of three virulence genes in diarrhoeal stool. Mol. Microbiol. **3:**1729–1734.
6. **Giron, J. A., M. S. Donnenberg, W. C. Martin, K. G. Jarvis, and J. B. Kaper.** 1993. Distribution of the bundle-forming pilus structural gene (*bfpA*) among enteropathogenic *Escherichia coli*. J. Infect. Dis. **168:**1037–1041.
7. **Giron, J. A., A. S. Y. Ho, and G. K. Schoolnik.** 1991. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science **254:**710–713.
8. **Gomez, T. A. T., V. Rassi, K. L. MacDonald, S. R. T. S. Ramos, L. R. Trabulsi, M. A. M. Vieira, B. E. C. Guth, J. A. N. Candeias, C. Ivey, M. R. F. Toledo, and P. A. Blake.** 1991. Enteropathogens associated with acute diarrheal disease in urban infants in São Paulo, Brazil. J. Infect. Dis. **164:**331–337.
9. **Guarino, A., M. Alessio, L. Tarallo, M. Fontana, G. Iacono, L. G. Casali, and S. Guandalini.** 1989. Heat stable enterotoxin produced by *Escherichia coli* in acute diarrhoea. Arch. Dis. Child. **64:**808–813.
10. **Gunzburg, S. T., N. G. Tornieporth, and L. W. Riley.** 1995. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. J. Clin. Microbiol. **33:**1375–1377.
11. **Hornes, E., Y. Wasteson, and O. Olsvik.** 1991. Detection of *Escherichia coli* heat-stable enterotoxin genes in pig stool specimens by an immobilized, colorimetric, nested polymerase chain reaction. J. Clin. Microbiol. **29:**2375–2379.
12. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155:**377–389.
13. **Levine, M. M., C. Ferreccio, V. Prado, et al.** 1993. Epidemiologic studies of *Escherichia coli* diarrheal infections in a low socioeconomic level peri-urban community in Santiago, Chile. Am. J. Epidemiol. **138:**849–869.
14. **Moseley, S. L., J. W. Hardy, M. I. Huq, P. Echeverria, and S. Falkow.** 1983. Isolation and nucleotide sequence determination of a gene encoding a heat-stable enterotoxin of *Escherichia coli*. Infect. Immun. **39:**1167–1174.

15. **Riley, L. W.** 1988. Infectious diseases associated with *Escherichia coli*, p. 237–261. *In* A. Balows, W. J. Hausler, M. Ohashi, and A. Turano (ed.), Laboratory diagnosis of infectious diseases. Principles and practice, 1st ed., vol. 1. Springer-Verlag, New York.
16. **Rolfs, A., I. Chuller, U. Finckh, and I. Weber Rolfs (ed.).** 1992. PCR: clinical diagnostics and research. Springer-Verlag, New York.
17. **Sack, D. A., S. Huda, P. K. B. Neogi, R. R. Daniel, and W. M. Spira.** 1980. Microtiter ganglioside enzyme-linked immunosorbent assay for vibrio and *Escherichia coli* heat-labile enterotoxins and antitoxin. J. Clin. Microbiol. **11:**35–40.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Scaletsky, I. C. A., M. L. M. Silva, and L. R. Trabulsi.** 1984. Distinctive patterns of adherence of enteropathogenic *Escherichia coli* to HeLa cells. Infect. Immun. **45:**534–536.
20. **Scotland, S. M., G. A. Willshaw, B. Said, H. R. Smith, and B. Rowe.** 1989. Identification of *Escherichia coli* that produces heat-stable enterotoxin ST_A by a commercially available enzyme-linked immunoassay and comparison of the assay with infant mouse and DNA probe tests. J. Clin. Microbiol. **27:** 1697–1699.
21. **Sethabutr, O., M. Venkatesan, G. S. Murphy, B. Eampokalap, C. W. Hoge, and P. Echeverria.** 1993. Detection of shigellae and enteroinvasive *Escherichia coli* by amplification of the invasion plasmid antigen H DNA sequence in patients with dysentery. J. Infect. Dis. **167:**458–461.
22. **Sommerfelt, H.** 1991. Nucleic acid hybridization for the identification of enterotoxigenic *Escherichia coli*. Rev. Med. Microbiol. **2:**138–146.
23. **Svennerholm, A. M., and J. Holmgren.** 1978. Identification of *Escherichia coli* heat-labile enterotoxin by means of a ganglioside immunosorbent assay (G_{M1}-ELISA) procedure. Curr. Microbiol. **1:**19–23.
24. **Venkatesan, M. M., J. M. Buysse, and D. J. Kopecko.** 1989. Use of *Shigella flexneri ipaC* and *ipaH* sequences for the general identification of *Shigella* spp. and enteroinvasive *Escherichia coli*. J. Clin. Microbiol. **27:**2687–2691.
25. **World Health Organization.** 1991. Programme for control of diarrheal diseases: interim programme report 1990. Document WHO/CDD/91.36. World Health Organization, Geneva.
26. **Yamamoto, T., T. Tamura, and T. Yokota.** 1984. Primary structure of heat-labile enterotoxin produced by *Escherichia coli* pathogenic for humans. J. Biol. Chem. **259:**5037–5044.